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Eduard A. Gorham - 10-19-97  
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### **Introduction:**

Chromosomal aberrations are found in all cancers that have been adequately studied. Highly specific aberrations often are associated with only one, or a small number, of tumor types. Thus karyotype analysis is useful in establishing a precise diagnosis. In addition, some aberrations arise during the later stages of disease and are associated with more aggressive malignancy. Therefore karyotype analysis also is useful as a prognostic indicator and for treatment planning.

Oncogenes or tumor suppressor genes are frequently observed to be located at the sites of tumor-specific chromosome aberrations, and are known to suffer mutation as a result of chromosomal rearrangement. These observations provide a firm scientific foundation for the clinical use of karyotype analysis. Furthermore, finding new tumor-specific chromosome aberrations is often the initial step in discovering new cancer genes. As such, karyotype analysis is an important tool for advancing our knowledge of carcinogenesis.

Despite their known association with cancer and birth defects, chromosomal inversions are perhaps the most difficult of all aberrations to detect on a routine basis. Therefore, it is doubtful that we know the full extent to which inversions are associated with cancer. It is the goal of this research project to develop a new tool for karyotype analysis that we call "chromatid painting". A chromatid paint is a complex mixture of labeled probes prepared using DNA cloned from a specific human chromosome. When hybridized to a metaphase chromosome spread, the probes anneal to multiple target sequences along the length of one chromatid of the chromosome. With fluorescence microscopy, the chromatid appears to be painted. A fluorescent DNA-binding dye counterstains the sister chromatid. The visual effect is that the two halves of the target chromosome fluoresce with different colors.

With a chromatid paint, an inversion will appear as a switch in labeling from one chromatid to the other within the inverted region. Since the pattern is easily recognized, chromatid paints will permit rapid identification of inversions. In addition, breakpoints can be localized precisely, and smaller inversions can be detected than is now possible using chromosome banding. Chromatid paints will be used to examine breast cancer cells for previously unidentified tumor-specific inversions. Any new aberrations revealed by this method will help to improve diagnostic and prognostic capabilities, and could be the beginning of an effort to identify genes at the breakpoints. For these reasons, we believe that chromatid paints will find application both in the clinic and in the laboratory.

### **Body:**

Award #DAMD17-97-1-7165 was the first U.S. Army-funded breast cancer research grant received at Los Alamos National Laboratory. Due to internal administrative difficulties, funds were not placed into a spendable account until February 1998. Therefore this annual report covers only the first 6½ months of effort on the project.

As stated in the Statement of Work, our first Technical Objective was to perfect the steps in preparing a chromatid paint according to the procedure described in the proposal. This procedure requires directional cloning of chromosomal DNA, PCR amplification, probe preparation, and fluorescence *in situ* hybridization (FISH) painting. The first Technical Objective was divided into tasks designed to test and optimize each step in the procedure. Our progress is reported below.

*Testing the adequacy of chromosomal DNA as a template for DNA polymerization.* The cloning procedure requires copying DNA from fixed cells attached to a glass substrate. Any step in the process potentially could damage the DNA and render it unsuitable as a template. A test

was devised for examining the adequacy of chromosomal DNA as a template for DNA polymerization. Chromosomal DNA was made single-stranded by the CO-FISH procedure (1). A short synthetic oligomer was then hybridized to a repetitive DNA sequence in the chromosomal DNA, and a primer extension reaction initiated. During DNA polymerization a fluorescently tagged nucleotide was incorporated into the newly synthesized DNA. This label made it possible to detect DNA polymerization by fluorescence microscopy. The procedure resembles a PRINS (primed *in situ* synthesis) reaction (2), but unlike PRINS the polymerization was expected to be strand-specific as indicated by a signal on one chromatid for tandem repeats. Two primers were utilized, one to telomeric DNA (TTAGGG)<sub>n</sub> and the other to a (AATGG)<sub>n</sub> repeat. Results demonstrated that chromosomal DNA prepared in this manner is indeed a suitable template for DNA synthesis (see appendix I). Also confirmed was the ‘one-sided’ nature expected of fluorescent signals from head-to-tail repetitive sequences (for example, chromosome 1 centromere targeted with the 5-mer) and two-sided signals from repeats that exist in a mixed orientation (chromosome 9 centromere). Using this testing procedure, we optimized our cell fixation protocol to maximize preservation of chromosomal DNA. Best results were achieved by initially fixing cells in methanol and adding acetic acid just before dropping cells onto coverslips. The procedure itself is a novel and useful addition to the techniques available to molecular cytogenetics.

*DNA amplification from small amounts of template.* The quantity of DNA expected to be available for PCR amplification is expected to be small, *i.e.* ~1pg. While successful PCR amplification starting from such small amounts of template has been reported previously, the procedure is not routine. In addition, PCR artifacts are potentially more troublesome in the preparation of chromatid paints, which need to be strand specific, as opposed to chromosome paints where strand specificity is not an issue. We therefore sought to optimize DNA amplification starting with 1 pg of a defined template. Analysis of the amplified product by gel electrophoresis allowed us to detect PCR artifacts. We learned that one of our PCR primers had a tendency to produce artifacts in blank (no template) reactions. A new primer was designed and tested with better results. Various PCR conditions (annealing temperature, number of cycles, buffer, etc.) were tested in order to optimize the reaction. We found that the type and quantity of DNA polymerase was important to achieving successful amplification of template without producing excessive PCR artifacts. Our best results were obtained by initializing the PCR amplification with a small amount of Pfu-turbo thermostable DNA polymerase and a low primer concentration. After 15 cycles additional primer was added to the reaction along with a larger quantity of the DNA polymerase Ampli-Taq Gold. This second polymerase activates gradually with each successive cycle. These amplification strategy was chosen because excessive polymerase and high primer concentrations have been shown to produce PCR artifacts. We can now successfully perform DNA amplification starting with very small template quantities.

*Directional cloning from cells on coverslip.* Our goal was to directionally clone chromosomal DNA, *i.e.* copy and amplify DNA segments from a single-stranded chromatid, according to the procedure described in the proposal. Hamster/human hybrid cells containing one human #11 chromosome were fixed and dropped onto microscope coverslips. These cells had been synchronized by mitotic shake-off so that the most were in the G<sub>1</sub> phase of the cell cycle. Chromosomal DNA was made single-stranded by the CO-FISH technique (1). Upstream (5') and downstream (3') oligomers were hybridized to the single-stranded chromatids. These oligomers contained flanking primer hybridization sites for later PCR amplification. DNA polymerization

and ligation joined the up- and down-stream oligomers. The up-stream oligomer had an Alu binding site allowing specificity for human DNA. Single cells were isolated on fragments of the coverslip and placed into a PCR tube for amplification. Analysis of the product by gel electrophoresis and quantification by spectrophotometry indicated successful cloning and amplification.

*Preparing and testing a chromatid paint.* A complex probe (paint) was prepared by performing a single-stranded DNA amplification using a single primer. The probe was labeled by incorporating the fluorescently tagged nucleotide, Cy3-dCTP, during amplification. The probe was painted (hybridized) to both human cells and the chromosome 11 hybrid cells. Early results showed that in the hybrid cells the human chromosome was clearly painted, while the hamster chromosomes showed little fluorescence. The human cells showed a contrast in fluorescence intensity between different chromosomes, but all were labeled to some extent. Of particular concern, these early experiments did not show differential fluorescence intensity between sister chromatids as would be expected of a chromatid paint. We attributed this negative result to three possible factors, cloning of some hamster DNA, Alu hybridization during painting that was not completely blocked by our painting protocol, and unintended priming during PCR amplification. We attempted to solve the first two potential problems by modifying our painting protocol to include blocking with human Cot 1 DNA, hamster Cot 1 DNA, and a synthetic oligomer having the same sequence as the Alu binding site in the up-stream oligomer. Our best results to date are shown in appendix II. While these results are encouraging, the paint will have to be considerably improved. The last problem could potentially link together cloned DNA segments into opposite orientations, thus destroying the strand specificity required of a chromatid paint. In order to minimize this problem, we have devised new strategies as discussed below.

*Microdissection approach to directional cloning.* Low template quantity has been shown to be a source of PCR artifacts. We have considered several strategies for increasing template quantity, and have chosen to pursue an approach based on chromosomal microdissection. Initial steps in the procedure, *i.e.* primer extension and ligation, are unchanged. However, in an additional step a centromeric probe is hybridized. This probe both identifies a specific chromosome and distinguishes between the two sister chromatids. Using microdissection several chromatids having the same DNA polarity can be scraped from cells attached to a coverslip and placed into a PCR tube. We have established a collaboration with Dr. Joel Bedford of Colorado State University, an expert in microdissection, to help us with this procedure. We also have taken steps to adapt an in-house micromanipulation facility for this purpose.

It is desirable (although not absolutely necessary) to microdissect and pool only chromatids that had not engaged in a sister chromatid exchange (SCE). For the CO-FISH procedure, normally cells are grown for one cell cycle in bromodeoxyuridine (BrdU). In these "first cycle cells" SCE can not be visualized. We therefore are working with cells grown in BrdU for two cycles in an attempt to distinguish between the singly and doubly substituted chromatids. Since it is desirable to simultaneously detect both the fluorescent probe and SCE, we counterstained with the fluorescent dye DAPI. It was observed that DAPI staining resulted in poor differentiation between sister chromatids. Another fluorescent dye, SYBR Green II, was tested. This dye shows enhanced fluorescence yield when bound to single-stranded DNA. The result was a very strong differentiation between sister chromatids (see appendix III). This procedure for simultaneously detecting fluorescent probes and SCE has to the best of our knowledge not been described before. In addition to serving our immediate needs, we have every reason to believe that it will be a useful

technique in its own right. We plan to continue pursuing the microdissection approach to creating a chromatid paint.

*Multiplex CO-PRINS approach to chromatid painting.* Motivated by our success with CO-PRINS, a new approach to chromatid painting was conceived. The procedure would work by performing a primer extension reaction from multiple points along a single-stranded chromatid. The chromatid becomes painted by incorporating a labeled nucleotide during DNA synthesis. To make the procedure work, numerous primers will need to be identified that are capable of priming DNA polymerization along the same chromosomal DNA strand. As a result, this method may be suitable only for chromosomes with extensive mapping and sequence data, chromosome 16 for example. Multiplex CO-PRINS has significant advantages such as not requiring directional cloning or PCR, the most difficult steps in our current protocol. The primary disadvantage is the need to design and synthesize numerous primers. We plan to test the feasibility of this approach by attempting to paint a portion of one arm of chromosome 16.

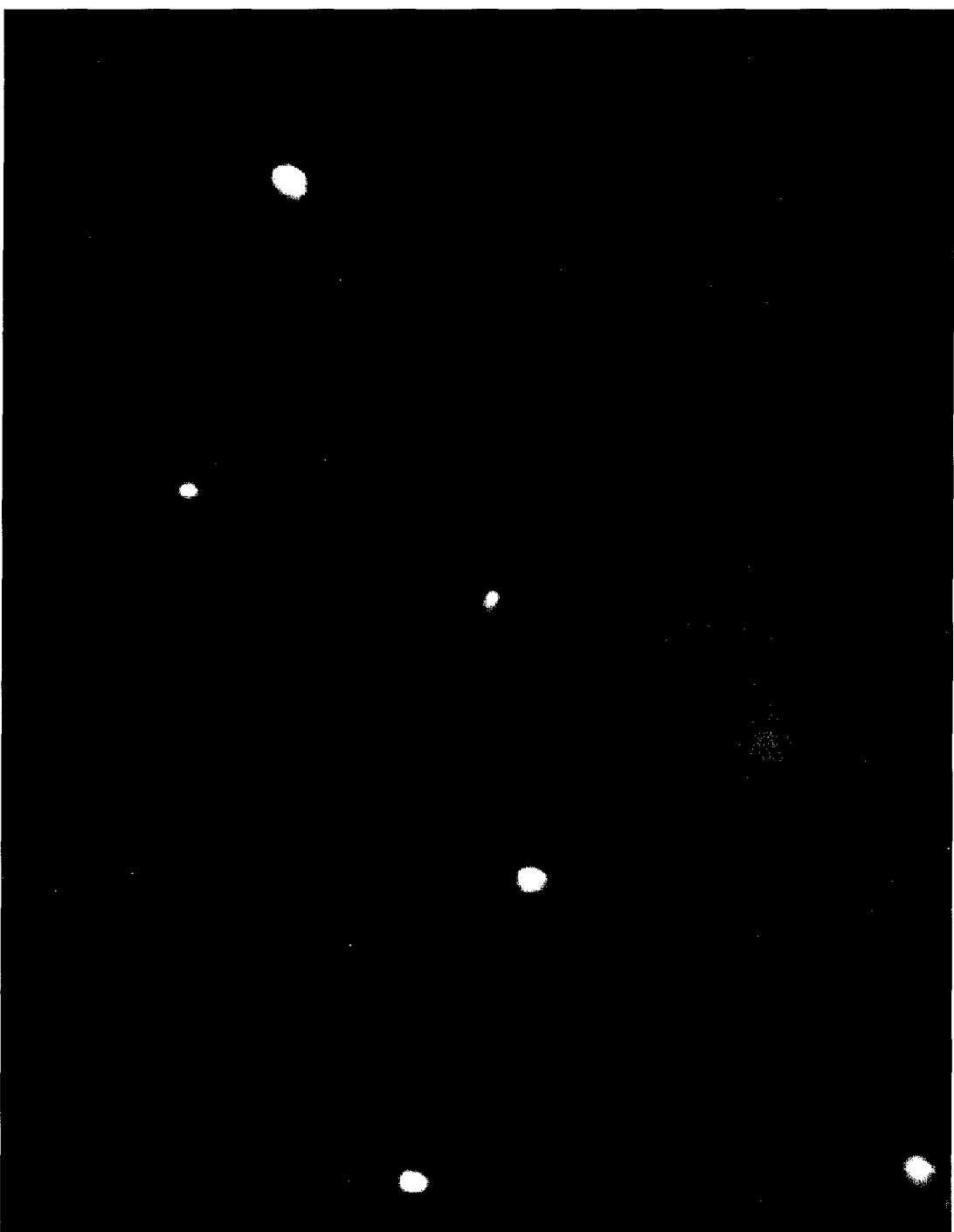
**Conclusions:** Significant progress has been made towards achieving Technical Objective 1 of our proposal. All of the steps required to prepare a chromatid paint have been shown to be feasible, although some require further optimization. We have demonstrated that 1) chromosomal DNA is not damaged during preparation procedures in a manner that would render it unsuitable as a template for DNA synthesis, 2) PCR amplification can be performed starting with very small quantities of template, 3) chromosomal DNA can be cloned by our methods, and 4) probes can be prepared from cloned and amplified DNA. As a byproduct of these efforts, two new and useful cytogenetic techniques were devised. An alternative approach to chromatid painting has been identified and will be examined more carefully in the future.

**References:**

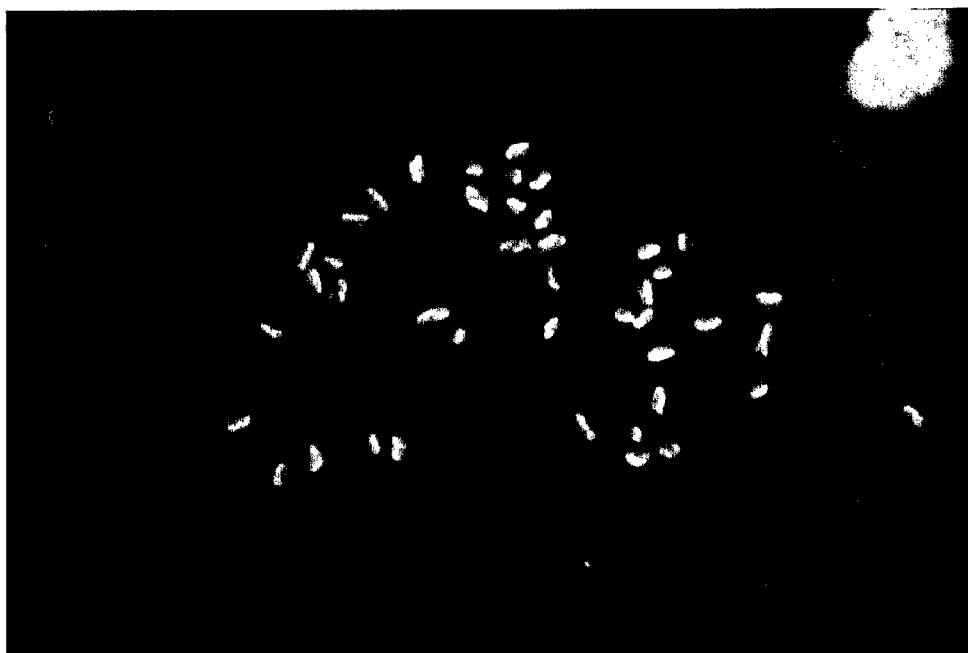
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- Koch JE, Kolvraa S, Petersen KB, Gregersen N and Bolund L. Oligonucleotide-priming methods for the chromosome-specific labelling of alpha satellite DNA in situ. *Chromosoma* 98, 259-265 (1989).

## Appendices

- I. Primer extension reaction on a single-stranded chromosomal DNA template (CO-PRINS). A synthetic oligomer primed extension along the (AATGG)<sub>n</sub> repetitive sequence in several chromosomes.
- II. Strand-specific hybridization of cloned DNA. Note asymmetrical signal intensity between chromatids, and a twist or SCE near the centromere.
- III. Combined CO-FISH and SCE detection. Top: Hybridization of a telomere probe to mouse cells. Bottom: Same cell stained with SYBR Green II shows differential staining, thus allowing identification of the singly and doubly substituted chromatids.









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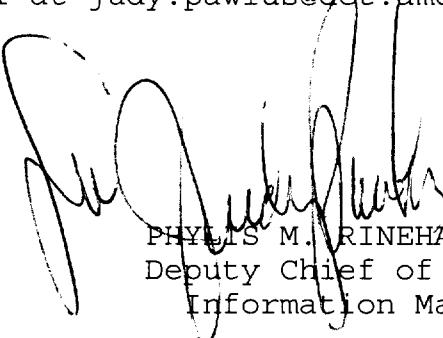
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